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Interaction with extracellular matrix proteins influences *Lsh/Ity/Bcg* (candidate *Nramp*) gene regulation of macrophage priming/activation for tumour necrosis factor-α and nitrite release

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SUMMARY

The murine resistance gene *Lsh/Ity/Bcg* regulates activation of macrophages for tumour necrosis factor-α (TNF-α)-dependent production of nitric oxide mediating antimicrobial activity against *Leishmania*, *Salmonella* and *Mycobacterium*. As *Lsh* is differentially expressed in macrophages from different tissue sites, experiments were performed to determine whether interaction with extracellular matrix (ECM) proteins would influence the macrophage TNF-α response. Plating of bone marrow-derived macrophages onto purified fibrinogen or fibronectin-rich L929 cell-derived matrices, but not onto mannan, was itself sufficient to stimulate TNF-α release, with significantly higher levels released from congenic B10.L-*Lsh*^{r} compared to C57BL/10ScSn (*Lsh*^{s}) macrophages. Only macrophages plated onto fibrinogen also released measurable levels of nitrites, again higher in *Lsh*^{r} compared to *Lsh*^{s} macrophages. Addition of interferon-γ (IFN-γ), but not bacterial lipopolysaccharide or mycobacterial lipoarabinomannan, as a second signal enhanced the TNF-α and nitrite responses of macrophages plated onto fibrinogen, particularly in the *Lsh*^{r} macrophages. Interaction with fibrinogen and fibronectin also primed macrophages for an enhanced TNF-α response to leishmanial parasites, but this was only translated into enhanced nitrite responses in the presence of IFN-γ. In these experiments, *Lsh*^{r} macrophages remained superior in their TNF-α responses throughout, but to a degree which reflected the magnitude of the difference observed on ECM alone. Hence, the specificity for the enhanced TNF-α responses of *Lsh*^{r} macrophages lay in their interaction with fibrinogen and fibronectin ECM, while a differential nitrite response was only observed with fibrinogen and/or IFN-γ. The results are discussed in relation to the possible function of the recently cloned candidate gene *Nramp*, which has structural identity to eukaryote transporters and an N-terminal cytoplasmic proline/serine-rich putative SH3 binding domain.

INTRODUCTION

The murine macrophage resistance gene *Lsh/Ity/Bcg* regulates the priming/activation of macrophages for antimicrobial activity against *Leishmania donovani*, *Salmonella typhimurium*, and various species of *Mycobacterium* including *M. bovis*, *M. lepraemurium* and *M. avium intracellulare*.[1–5] Recently Vidal and co-workers[6] isolated a candidate gene, designated *Nramp* (natural resistance-associated macrophage protein), which encodes a polytopic integral membrane protein that has structural features common to prokaryotic and eukaryotic transporters. The presence of a small consensus motif showing sequence identity with nitrate transporters led these workers to hypothesize that *Nramp* might be involved in direct delivery of nitrates to the phagolysosome of the infected macrophage, where the acid environment would mediate conversion via nitrites to toxic nitric oxide (NO). However, this hypothesis fails to take account of the many studies demonstrating that *Lsh/Ity/Bcg* regulates priming/activation which, although ultimately resulting in the tumour necrosis factor-α (TNF-α)-dependent production of antimicrobial NO,[7] has been shown to have many pleiotropic effects[1,2,4,5,8,9] including: (1) down-regulation of 5′nucleotidase; (2) up-regulation of major histocompatibility complex (MHC) class II, TNF-α production, interleukin-1β (IL-1β) expression, AcM.1 antigen expression, oxidative burst and tumoricidal activity; and (3) rapid (within 30 min) up-regulation of the early gene KC, a neutrophil-specific chemoattractant belonging to the IL-8-related C-X-C family of small peptide cytokines, in response to mycobacterial lipoarabinomannan (LAM).[10]

Our laboratory has recently isolated and sequenced a series of full-length *Nramp* clones from an activated B10.L-*Lsh*^{r} macrophage cDNA library.[11] These clones all differed in the 5′ region from the published[6] pre-B-cell-derived clone sequence and result in the addition of 64 amino acids at the N-terminus

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of the predicted protein. This new cytoplasmic domain is rich in proline, serine and basic amino acids, and contains three protein kinase C phosphorylation sites (in addition to the two already identified in the published sequence), as well as a putative Src homology 3 (SH3) binding domain. RNA containing this extra domain are the only form found within the macrophage. SH3 domains occur as related sequences in tyrosine kinases[12] and are believed to mediate protein–protein interactions obligatory for signal transduction.[13,14] Hence, this new sequence may be important in signal transduction for macrophage priming/activation and/or in regulating the transport function of *Nramp*.

One observation which has remained consistent throughout our earlier functional studies has been the variable expression of *Lsh* gene activity against *L. donovani*, *L. mexicana* or *L. major* in different macrophage populations both *in vivo* and *in vitro*.[1,7,15–18] Of particular interest is the inability of resident peritoneal or splenic macrophages from congenic Lsh^r and Lsh^s mice to exhibit differences in anti-leishmanial activity,[17] while resident tissue macrophages (e.g. Kupffer cells, lung macrophages) appear to be already primed for differential anti-leishmanial activity when isolated and infected with *L. donovani in vitro*.[17] This suggests that the tissue localization of macrophages and their different interactions with extracellular matrix (ECM) proteins might influence their activation state. Other work has shown, for example, that adherence of human alveolar macrophages[19] to collagen- or fibronectin-coated plates induced expression of mRNA for the chemotactic cytokine IL-8, as well as the proinflammatory cytokines IL-1β and TNF-α, and that the 120,000 MW cell-binding fragment of fibronectin was capable of stimulating TNF-α secretion by human monocytes.[20] In the latter case, TNF-α secretion induced by intact fibronectin was partially (50%) inhibitable by the addition of the peptide GRGDSP, suggesting a role for the common RGD binding site of the integrin receptor $\alpha_5\beta_1$, which mediates binding of fibronectin to macrophages,[21] in eliciting TNF-α release. As the autocrine activity of TNF-α released during interferon-γ (IFN-γ)/lipopolysaccharide (LPS) priming/activation of *Lsh*-resistant macrophages is crucial for antimicrobial activity,[7] experiments were undertaken to determine whether interaction with ECM influences the macrophage response. This would also be consistent with previous studies[22] demonstrating a role for tyrosine kinases in integrin-mediated signal transduction for TNF-α release. Here we present evidence that interaction with the ECM proteins fibrinogen and fibronectin specifically and preferentially primes macrophages from Lsh^r mice for enhanced TNF-α release, with interesting consequences for release of the stable nitrite end-product of NO production.

MATERIALS AND METHODS

Mice

N10 congenic B10.L-Lsh^r[23] and C57BL/10ScSn (= B10) (Lsh^s) were bred and housed under conventional animal house conditions. Mice (6–12 weeks) were matched by age (±2 weeks) and sex within each experiment.

Media and reagents

RPMI (Dutch Modification, ICN Flow Laboratories, Irvine, U.K.) or DMEM (cat. no. 10-331-26; ICN Flow Laboratories) were supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0·05 mM 2-mercaptoethanol (2-ME), and 10% fetal calf serum (FCS) (endotoxin and mycoplasma negative; Sigma Chemical Co., Pool, U.K.). *Salmonella typhimurium* LPS (L6143, phenolic extract, tissue culture grade), mannan (M3640) and fibrinogen (F4385) were obtained from Sigma. LPS was used at 0·1 ng/ml. LAM was generated in the endotoxin-free form by Dr D. Chatterjee and colleagues (Department of Microbiology, Colorado State University, Fort Collins, CO) as previously described.[24] Arabinose-rich AraLAM from the attenuated Colorado strain 'H37Ra'[24] were compared with the extensively mannosylated ManLAM from the virulent Erdman strain. The levels of contaminating endotoxin in the LAM preparations were determined using a quantitative Limulus amoebocyte lysate commercial assay (E-Toxnate, Sigma Kit no. 210; Sigma, St Louis, MO). As reported previously,[10] the batches of AraLAM and ManLAM used here contained 0·0108 and 0·0268 ng endotoxin/μg of LAM respectively, which did not confer ability to induce either TNF-α or early response gene (KC) expression on the non-stimulatory ManLAM preparation. LAM were used at 1000 ng/ml. Murine recombinant IFN-γ was obtained through Gunter Adolf of Boehringer Ingelheim (Ingelheim, Germany) and used at 25 U/ml. Monoclonal anti-mouse TNF-α (TN3) was kindly provided by R. Shreiber (Department of Pathology, Washington University Medical School, St Louis, MO). Rabbit anti-mouse TNF-α for the enzyme-linked immunosorbent assay (ELISA) was produced in-house. Neutralizing rabbit anti-mouse TNF-α used *in vitro* was kindly provided by Dr J. Tite (Wellcome Research Laboratories, Beckenham, U.K.).

Parasites

Leishmania donovani (LV9) and *L. major* (LV39) promastigotes recently transformed from amastigotes (maximum two subcultures) were grown in Dulbecco's modified Eagle's minimal essential medicine (DMEM) with 10% FCS and harvested at day 10/11 (stationary phase = metacyclic) for macrophage experiments. The proportion of logarithmic (PNA^+) versus metacyclic (PNA^-) in the cultures was determined using fluorescein isothiocyanate (FITC)–peanut agglutanin as described.[25] Day 10/11 cultures of *L. donovani* or *L. major* routinely contained 80–90% PNA^- parasites with metacyclic morphology. Metacyclic promastigotes were used at a ratio of 5 : 1 parasites : macrophages.

Coating of plates

The wells of flat-bottom 96-well tissue culture plates (Linbro, Flow Laboratories) were coated with fibrinogen at 0·1 μg/well in 0·2 M carbonate/bicarbonate buffer (pH 9·6). Plates were coated for 24 hr at 4°. Unbound fibrinogen was removed by washing three times in RPMI. Mannan coating of wells was by a modification of the method of Michl *et al.*[26] Briefly, 50 μl/well poly-L-lysine (0·1 mg/ml) was added to 96-well plates and left for 30 min. Plates were washed thoroughly in phosphate-buffered saline (PBS) and 100 μl/well 2·5% glutaraldehyde added for 15 min. Plates were washed thoroughly and mannan added at 2·5 mg/ml for 24 hr at room temperature. Washing three times with PBS was followed by the addition of 100 μl/well glycine at 15 mg/ml. Plates were left overnight and then washed three times with PBS prior to plating of macrophages.

ECM-coated plates rich in fibronectin were produced by culture (3 days at 37° in 10% FCS/RPMI) and later removal (1 M EDTA, pH 7) of L929 fibroblast cells (10^4/well) on 96-well plates prior to plating of macrophages. The fibroblasts secrete ECM rich in fibronectin onto the wells. This matrix will be referred to as fibronectin throughout, although other ECM (e.g. collagens) may be present. Control wells were left uncoated or treated with glutaraldehyde, as appropriate.

Bone marrow-derived macrophages

Mature bone marrow-derived macrophages were grown in DMEM on bacterial plastic Petri dishes (Bibby Sterilin Ltd, Stone, U.K.) as described previously,[7] harvested and replated in RPMI at 10^5 cells/well into 96-well plates coated with mannan, fibronectin, fibrinogen or control. Macrophages were incubated at 37° for 1 hr to adhere. Soluble stimuli (LPS, IFN-γ, LAM) were added simultaneously and parasites were added 2 hr after IFN-γ. Supernatants for TNF-α or nitrite assays were collected at 24 hr.

TNF-α ELISA

TNF-α in culture supernatants was measured by capture ELISA using the monoclonal antibody TN3 (10 μg/well) to coat 96-well plates (Nunc-immunoplate maxisorb, Nunc, Roskilde, Denmark) and the rabbit anti-TNF-α polyclonal (1:200) for second layer detection. Goat anti-rabbit IgG (H and L chains) peroxidase conujugate was used and substrate, 2,2′-azino-di-[3-ethyl-benzthiazoline sulphonate] (ABTS) with hydrogen peroxide (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). Supernatants were titrated from neat, 1 : 3, 1 : 9 and 1 : 27 and TNF-α U/ml calculated from a standard curve (2·5 to 50 U/ml) of rTNF-α run on each plate.

Measurement of nitrite release

Griess reagent: 1% sulphanilamide (Sigma), 0·1% napthylethylenediamine hydrochloride (Sigma) and 2·5% orthophosphoric acid (Analar BDH, Dagenham, U.K.) was freshly made prior to use. Griess reagent was added 1:1 with supernatant and left 5 min at room temperature. Standards were prepared using sodium nitrite (2–100 μM) and were included on each assay plate. Absorbances at 570 nm were read on a spectrophotometer (Dynatech, Billingshurst, U.K. or Multiscan, ICN Flow, High Wycombe, U.K.).

Statistical analysis

All assays were performed in quadruplicate. Within experiment variation in the response is indicated as mean ± SD for the quadruplicate readings. The Student's *t*-test was used to determine significant differences between Lsh^r and Lsh^s macrophages within experiments. A non-parametric Wilcoxon paired comparison test was used to determine whether differences between macrophages from Lsh^r versus Lsh^s mice remained significant over multiple treatments and multiple experiments. Results of these statistical analyses are incorporated into the figure legends.

RESULTS

TNF-α response in macrophages plated onto different matrices

Figure 1a demonstrates that the plating of bone marrow-derived macrophages onto the ECM proteins fibronectin and fibrinogen, but not onto mannan, is itself sufficient to stimulate TNF-α release. Significantly higher levels of TNF-α were released by macrophages from Lsh^r compared to Lsh^s mice. Only macrophages plated onto fibrinogen also produced measurable nitrite levels (Fig. 1b), suggesting that ligation of receptors binding this matrix (but not fibronectin) provided signals for both TNF-α release and induction of inducible NO synthase (iNOS) activity.

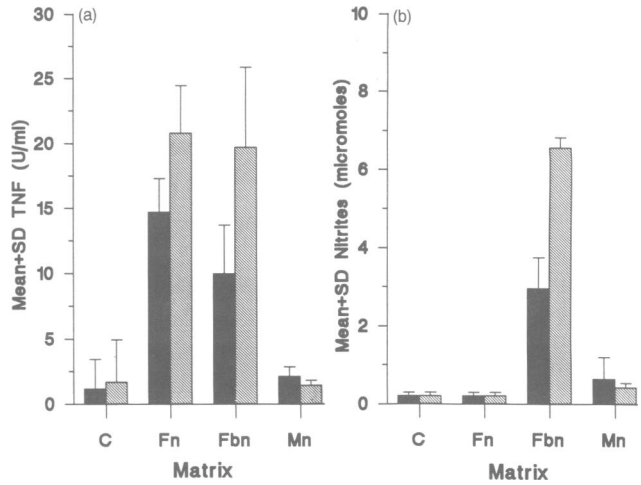

Figure 1. TNF-α (a) and nitrite (b) release for macrophages plated onto fibrinogen (Fbn), fibronectin (Fn) or mannan (Mn). Significant differences between Lsh^r (▨) and Lsh^s (■) macrophages were observed for TNF-α on fibrinogen (*t*-test: $0{\cdot}01 < P < 0{\cdot}025$) and fibronectin (*t*-test: $0{\cdot}01 < P < 0{\cdot}025$), and for nitrite release on fibrinogen (*t*-test: $P < 0{\cdot}0005$). Differences in TNF-α release for macrophages plated onto ECM remained significant over three independent experiments (Wilcoxon paired comparison test: $P < 0{\cdot}01$).

Fibrinogen primes for an enhanced response to IFN-γ but not to low-dose LPS

Addition of a second signal of IFN-γ enhanced the TNF-α response of macrophages plated onto fibrinogen (Fig. 2a). This was particularly pronounced in the Lsh^r macrophages. LPS at 0·1 ng/ml did not stimulate TNF-α release above that already elicited by plating onto fibrinogen, and IFN-γ plus LPS caused the same level of response as IFN-γ alone. Nitrite release (Fig. 2b) was also enhanced by addition of IFN-γ, with Lsh^r macrophages maintaining an advantage throughout all treatments. In control macrophages, IFN-γ alone did not stimulate TNF-α release but did elicit measurable nitrite release, which was significantly higher in Lsh^r compared to Lsh^s macrophages. IFN-γ also synergized with low-dose LPS for enhanced TNF-α and nitrite responses, but these were equivalent in macrophages from the two mouse strains. Although plating of macrophages onto mannan-coated wells did not itself elicit TNF-α or nitrite release, it did prime for an enhanced response to IFN-γ and, to a lesser extent, LPS. In this case, IFN-γ and LPS together had an additive effect. For macrophages plated on mannan, the response was equivalent for Lsh^r and Lsh^s throughout all treatments.

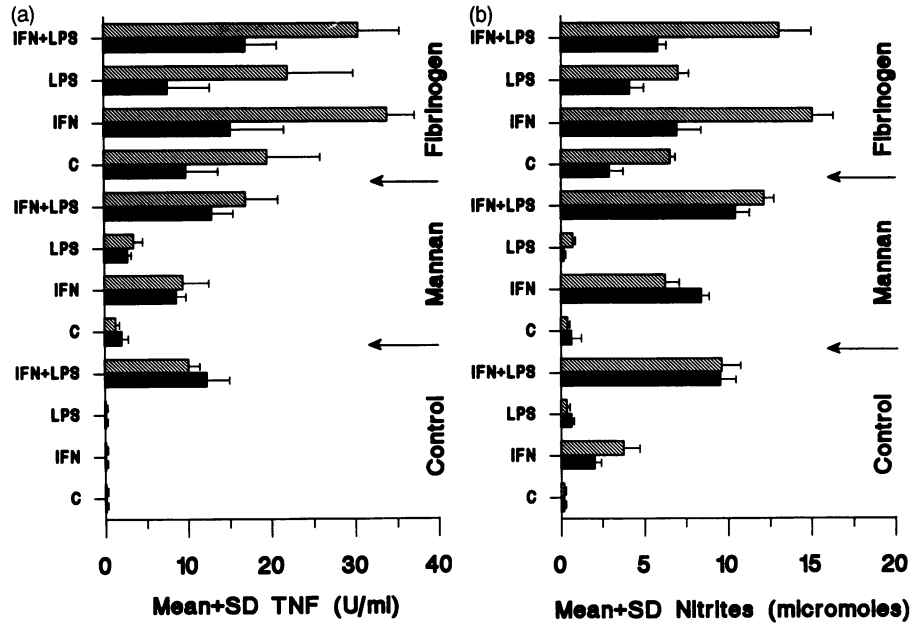

Figure 2. TNF-α (a) and nitrite (b) release for macrophages [Lsh^r (▨); Lsh^s (■)] plated onto fibrinogen or mannan, with second signals of IFN-γ and/or LPS. Differences in TNF-α and nitrite release remained significantly higher in Lsh^r macrophages plated on fibrinogen over all treatments (Wilcoxon paired comparison test: TNF-α, $P < 0{\cdot}005$; nitrites, $P < 0{\cdot}005$). A significant difference in nitrite release in response to IFN-γ was also observed for control macrophages (t-test: $0{\cdot}01 < P < 0{\cdot}025$).

Fibrinogen and fibronectin prime for enhanced TNF-α response to leishmanial parasites

As the *Lsh* gene acts independently of a functional T-cell population (and hence of a major source of IFN-γ) to control early growth of the parasite population, we were interested to determine whether interaction of the macrophages with specific ECM proteins would prime them for an enhanced TNF-α and/or nitrite responses to leishmanial parasites. Figure 3 shows that interaction with fibrinogen primes macrophages for an enhanced response to *L. major* and *L. donovani* metacyclic promastigotes equivalent to that seen with IFN-γ. IFN-γ plus

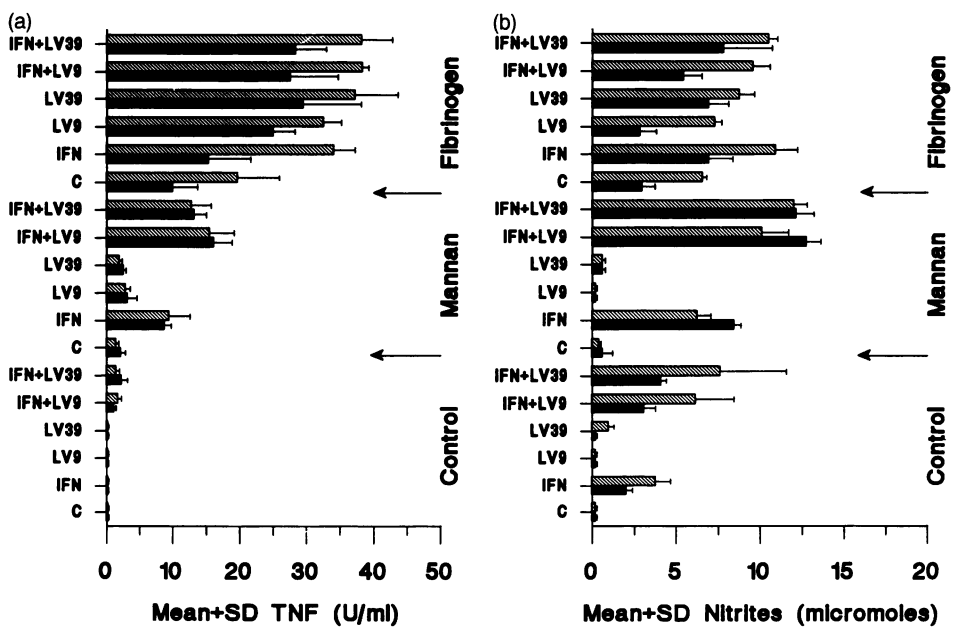

Figure 3. TNF-α (a) and nitrite (b) release for macrophages [Lsh^r (▨); Lsh^s (■)] plated onto fibrinogen or mannan, with second signals of IFN-γ and/or LV9 or LV39 metacyclic parasites. Differences in TNF-α and nitrite release remained significantly higher in Lsh^r macrophages plated on fibrinogen over all treatments (Wilcoxon paired comparison test: TNF-α, $P < 0{\cdot}01$; nitrites, $P < 0{\cdot}01$). As noted in Fig. 2 (same experiment), a significant difference in nitrite release in response to IFN-γ was also observed for control macrophages.

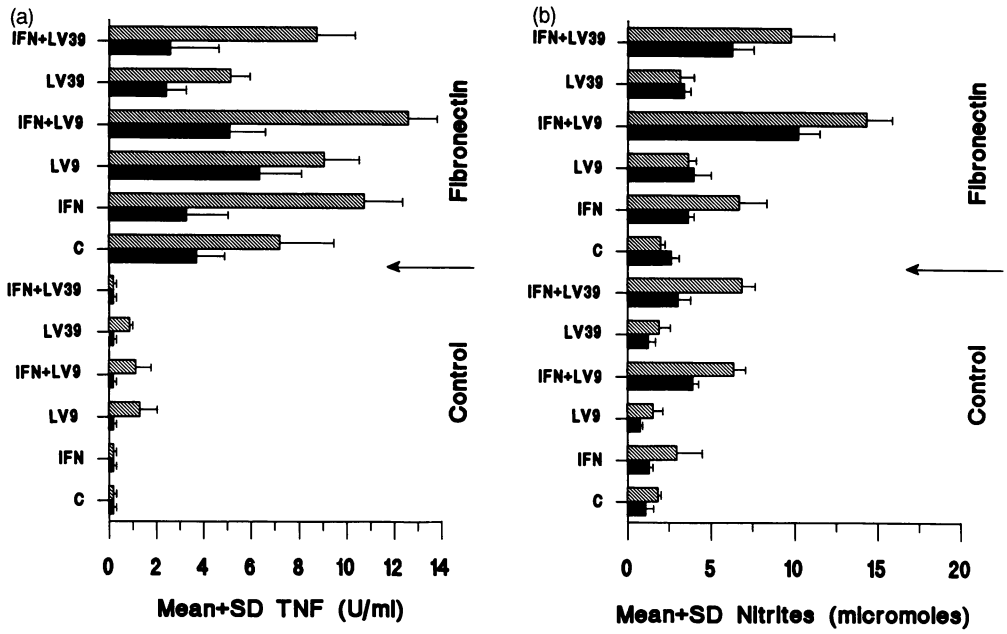

Figure 4. TNF-α (a) and nitrite (b) release for macrophages [*Lsh*^{r} (▨); *Lsh*^{s} (■)] plated onto fibronectin, with second signals of IFN-γ and/or LV9 or LV39 metacyclic parasites. Differences in TNF-α release remained significantly higher in *Lsh*^{r} macrophages plated on fibronectin over all treatments (Wilcoxon paired comparison test: $P < 0{\cdot}005$). In contrast, significant differences in nitrite release were only observed in the presence of IFN-γ (*t*-tests: IFN-γ alone, $0{\cdot}01 < P < 0{\cdot}025$; IFN-γ + LV9, $0{\cdot}01 < P < 0{\cdot}025$; IFN-γ + LV39, $0{\cdot}025 < P < 0{\cdot}05$). As observed in Fig. 2 (different experiment), a significant difference in nitrite release in response to IFN-γ was also observed for control macrophages (*t*-test: $P = 0{\cdot}05$).

metacyclic parasites was not greater than either stimulus alone, although this may simply reflect the fact that the response was already maximal. For these macrophages plated onto fibrinogen, *Lsh*^{r} macrophages remained superior in their response throughout but to a degree which reflected the magnitude of the difference observed on fibrinogen alone. Hence, metacyclic parasites themselves did not appear to provide a differential signal for *Lsh*^{r} versus *Lsh*^{s} macrophages. Nor did they provide a second signal for enhanced nitrite release above the background of plating onto fibrinogen (Fig. 3b). In the control macrophages plated onto uncoated wells, IFN-γ alone, parasites alone, and IFN-γ plus parasites did not stimulate significant TNF-α release, although IFN-γ alone and in combination with parasites did stimulate measurable nitrite release greater in *Lsh*^{r} than *Lsh*^{s} mice (Fig. 3b). Macrophages plated onto mannan (Fig. 3a) showed slightly enhanced TNF-α responses to parasites alone. In this case, IFN-γ plus parasites was additive, and IFN-γ alone or in combination with parasites stimulated nitrite release (Fig. 3b). For macrophages on mannan, the response of *Lsh*^{r} and *Lsh*^{s} mice was equivalent throughout.

In other experiments, the response of macrophages plated onto fibronectin was examined using metacyclic parasite populations (Fig. 4). As previously noted (Fig. 1), plating onto fibronectin caused enhanced TNF-α release, greater in *Lsh*^{r} than in *Lsh*^{s} macrophages, which was further enhanced in *Lsh*^{r} macrophages by addition of IFN-γ (Fig. 4a). As before, metacyclic parasites elicited minimal TNF-α release in control macrophages even with IFN-γ priming. Neither *L. donovani* nor *L. major* metacyclic promastigotes enhanced TNF-α production above that induced by plating onto fibronectin, but the *Lsh*^{r} macrophages remained superior in their TNF-α response for all combinations of parasites with/without IFN-γ. Interestingly, the enhanced TNF-α signal observed by plating macrophages onto fibronectin was only translated into enhanced nitrite production in the presence of IFN-γ (Fig. 4b), and a difference between *Lsh*^{r} and *Lsh*^{s} macrophages was also only observed in the presence of IFN-γ. Hence, fibronectin itself or in combination with parasites did not provide a second signal for up-regulation of iNOS activity, although parasites did act synergistically with IFN-γ for both control and macrophages plated onto fibronectin. Although addition of neutralizing anti-TNF-α antibody inhibited TNF-α production in IFN-γ-stimulated fibronectin-plated macrophages by >90% (98% *Lsh*^{r}; 93% *Lsh*^{s}), the nitrite response was inhibited by only 52% and 56% for *Lsh*^{r} and *Lsh*^{s} macrophages, respectively. Taken together these results indicate that the TNF-α elicited by plating onto fibronectin acts synergistically with IFN-γ for nitrite release but cannot act alone.

Mannan but not fibrinogen primes for enhanced response to mycobacterial LAM

In previous studies we[10] and others[24] have shown that ManLAM and AraLAM from virulent and attenuated *M. tuberculosis* differ in their ability to elicit a TNF-α response. Orme and co-workers[27] had also shown that *Lsh*-controlled resistance was not observed when highly virulent strains of *M. bovis*, which have recently been shown to contain a high proportion of ManLAM,[28,29] are inoculated into resistant and susceptible mouse strains *in vivo*. This would be consistent with the observation that the *Lsh* resistance mechanism requires the stimulation of a TNF-α response. Experiments were therefore performed to determine whether interaction with fibrinogen or

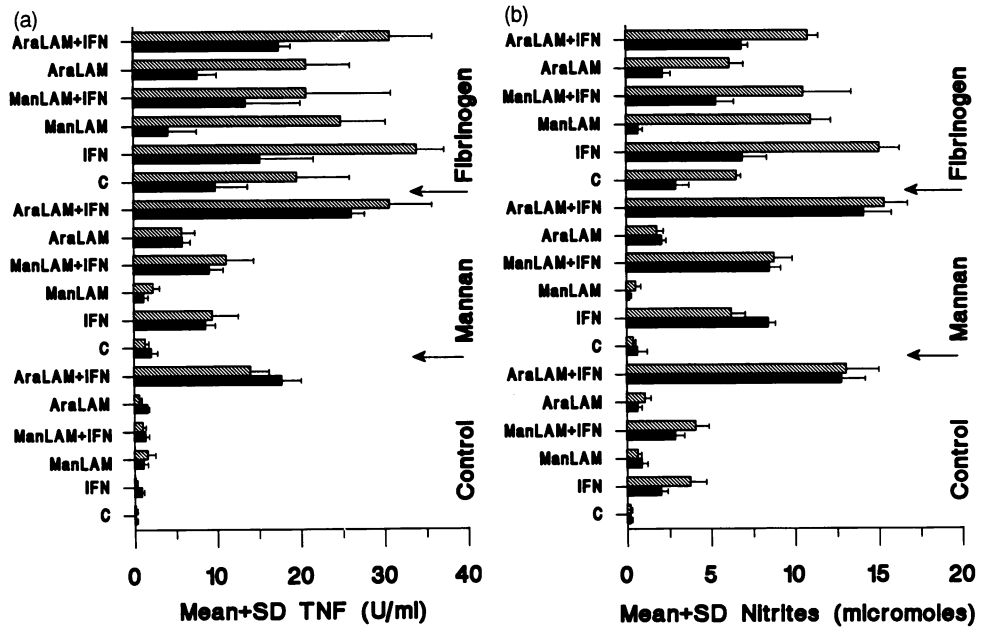

Figure 5. TNF-α (a) and nitrite (b) release for macrophages [*Lsh*^{r} (▨); *Lsh*^{s} (■)] plated onto fibrinogen or mannan, with second signals of IFN-γ and/or AraLAM or ManLAM. Differences in TNF-α and nitrite release remained significantly higher in *Lsh*^{r} macrophages plated on fibrinogen over all treatments (Wilcoxon paired comparison test: TNF-α, $P < 0{\cdot}005$; nitrites, $P < 0{\cdot}005$). As noted in Fig. 2 (same experiment), a significant difference in nitrite release in response to IFN-γ was also observed for control macrophages.

mannan influenced the macrophage response to mycobacterial LAM. As with the previous studies, ManLAM and AraLAM at 1000 ng/ml failed to elicit a TNF-α response in control macrophages (Fig. 5a). In combination with IFN-γ, AraLAM but not ManLAM stimulated a significant TNF-α response which was equivalent in control macrophages from *Lsh*^{s} and *Lsh*^{r} mice. As before, IFN-γ alone induced nitrite release greater in *Lsh*^{r} then *Lsh*^{s} macrophages, but equivalent when in synergy with AraLAM. Plating of macrophages onto mannan-primed macrophages for an additive enhanced TNF-α response to IFN-γ and to AraLAM, but not to ManLAM. On mannan, this enhanced TNF-α release was not translated into nitrite release unless IFN-γ was present. As before, equivalent responses were observed for *Lsh*^{r} and *Lsh*^{s} for all responses of mannan-plated macrophages. Macrophages plated onto fibrinogen, whilst maintaining the usual enhanced TNF-α response in *Lsh*^{r} over *Lsh*^{s} macrophages, did not respond further to either AraLAM or ManLAM with or without addition of IFN-γ. On fibrinogen, all of the TNF-α responses were translated into enhanced nitrite release with/without LAM even in the absence of IFN-γ, suggesting again that ligation of fibrinogen provides a direct signal for induction of iNOS and hence enhanced nitrite release.

DISCUSSION

Results presented here demonstrate that plating of bone marrow-derived macrophages onto the ECM proteins fibrinogen and fibronectin specifically and preferentially stimulates macrophages from the congenic B10.L-*Lsh*^{r} mouse strain for enhanced TNF-α release when compared to the susceptible B10 (*Lsh*^{s}) strain. The specificity of this integrin-mediated interaction in influencing the *Lsh* gene regulated pathway of macrophage activation was reinforced by the observation that ligation of the mannose fucose receptor (MFR) by plating macrophages onto immobilized mannan did not itself elicit a TNF-α response, and did not prime for a differential response to other stimuli in *Lsh*^{r} versus *Lsh*^{s} macrophages. The differential response observed in macrophages from the *Lsh* congenic mouse strains following interaction with ECM proteins may help to explain the variable expression of the *Lsh* resistance phenotype in macrophages from different tissue sites,[17] especially for liver Kupffer cells which have been shown to bind fibronectin with high affinity.[30] This process of ECM-mediated differences in *Lsh* gene activity would be consistent with the many independent observations now being made on the important role of ECM in determining tissue-specific activity of genes involved in a wide range of functions.[33] With respect to the macrophage response, our results support earlier studies[19,20,32] demonstrating that adherence of human macrophages to ECM stimulates TNF-α, in addition to IL-8, IL-1β and immediate early gene responses. Perhaps most significantly, our data provide strong support for the hypothesis that *Nramp* mediates differential priming/activation of macrophages from susceptible and resistant mouse strains by acting as a co-stimulatory molecule in integrin-mediated tyrosine kinase signal transduction pathways.[11] As IFN-γ alone is known to prime for differential MHC class II expression in *Lsh*^{r} versus *Lsh*^{s} macrophages,[33,34] and was here shown to elicit differential nitrite release, it is possible that *Nramp* may also act as a co-stimulatory molecule in IFN-γ-mediated amplification of signals in resistant macrophages. This would be consistent with recent studies demonstrating the importance of tyrosine–phosphorylation events in IFN-γ-induced macrophage responses.[35]

In experiments presented here amplification of the priming/activation signal in resistant macrophages was measured as a change in the TNF-α response, a step we have previously

considered is essential to the enhanced antimicrobial activity of IFN-γ/LPS-activated resistant macrophages.[7] Whether this amplified signal is translated into differential antimicrobial activity may, however, be dependent on the presence of a second signal for up-regulation of iNOS and generation of a threshold level of NO required for kill.[7] Recent analysis of the promoter region of the murine iNOS gene has, in fact, identified a complex array of regulatory elements including 10 copies of an IFN-γ response element, three copies of a γ-activated site, two copies each of a nuclear factor-κB site, an IFN-α-stimulated response element, an activating protein 1 site, a TNF-α response element and one X-box,[36] suggesting that the regulation of expression of iNOS may rely on complex interactions between stimuli. Plating of macrophages onto different ECM may directly or indirectly stimulate factors binding to one or more of these response elements. For example, in experiments presented here plating of macrophages onto fibrinogen was sufficient to elicit both TNF-α and nitrite release, whereas macrophages on fibronectin required a second IFN-γ signal for nitrite production. It was interesting too that leishmanial parasites failed to enhance either TNF-α or nitrite responses, whereas mycobacterial AraLAM was a potent co-stimulator for both. To begin to unravel the complexity of these interactions and, in particular, the specific receptor/ligand events important in *Lsh* gene-regulated TNF-α responses, it is of interest to consider further the specificity of the receptor interactions known to occur between ECM/LPS/LAM/leishmanial parasites and macrophages.

Interaction of fibronectin with macrophages is via members of the β_1 integrin family, principally $\alpha_5\beta_1$ (VLA-5[37–39]). There is ample evidence from previous studies that β_1 integrins are involved in tyrosine kinase signal transduction for altered gene expression.[40–42] Fibrinogen has also recently been shown to bind to a myeloblast cell line through a β_1-class integrin,[43] although it is more commonly believed to bind to cells of the myeloid lineage through the β_2 integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18; the macrophage type three complement receptor CR3).[44–46] Previous studies[47] using specific monoclonal antibody (M1/70) stimulation have demonstrated that Mac-1 can act as an activation receptor on macrophages, priming for enhanced respiratory burst activity and up-regulated surface expression of MHC class II molecules. M1/70 has also previously been shown to prime differentially resident peritoneal macrophages from congenic *Lsh*^{r} and *Lsh*^{s} mice for enhanced phorbol 12-myristate 13-acetate (PMA)-elicited superoxide production.[23] Leishmanial promastigotes and mycobacterial LAM also bind to CR3, as well as to the MFR.[25,48,49] Neither were capable, however, of directly (we did not test their ability to prime) stimulating TNF-α release in control macrophages, or of differentially enhancing the response in macrophages plated onto ECM. The latter may simply reflect the fact that plating macrophages onto fibrinogen would remove CR3 from the macrophage surface. This would leave parasite interaction with MFR as an alternative route to enhanced TNF-α secretion which, as our results with ManLAM and mannan-coated surfaces clearly demonstrate, would not signal for preferentially enhanced TNF-α release in *Lsh*^{r} macrophages. It is possible, too, that cross-linking[32,50] or some other form of interaction between the β_1 and β_2 integrins, which would not be provided by the parasite or LAM stimuli, is important in triggering the *Nramp*-mediated differential response.

Ligation of fibronectin to the $\alpha_5\beta_1$ integrin on macrophages has been shown to alter the avidity (and hence the biological activity) of CR3.[51] Hence, the effects of fibronectin on macrophage function could be mediated secondarily through the β_2 integrins. In experiments performed here there was, however, no requirement for a second signal via Mac-1 to obtain enhanced TNF-α release in *Lsh*^{r} macrophages following plating onto fibronectin. Indeed, a second (parasite) signal via Mac-1 did not enhance the TNF-α response, and did not provide a second signal for nitrite release. This suggests that interaction with the β_1 integrins is sufficient to obtain a differential *Nramp*-related TNF-α response, but this will not be translated into the effector for enhanced antimicrobial activity without a second (e.g. IFN-γ) signal.

Conversely, activation of macrophages can lead to enhanced secretion of fibronectin by macrophages,[52] which may contribute to β_1-mediated induction of inflammatory mediators. We do not know whether plating of macrophages onto fibrinogen itself induces fibronectin secretion. In any case, the ability of fibrinogen to bind to β_1 integrins[43] may be sufficient to mediate the differential *Nramp*-related response. The observation that LPS mediates fibronectin secretion[52] may, however, help to explain discrepencies between results presented here and those of our earlier work.[1] In experiments presented here we failed to see dramatic *Lsh* gene-regulated differences in TNF-α release by control macrophages after IFN-γ/LPS stimulation whereas the earlier work[1] showed a clear enhancement of the response in macrophages from *Lsh*^{r} mice. In the earlier experiments[1] bone marrow macrophage populations were expanded in medium containing horse serum rich in LPS, which could influence the activation state of the macrophages either directly through interaction with β_2 integrins or indirectly through a β_1 integrin/fibronectin response.[44,51] In the more recent experiments (this study and refs 7 and 53) we have rigidly adhered to a protocol for expansion of the bone marrow macrophage population under strictly endotoxin-free conditions. It was interesting too that, in the earlier experiments,[1] preincubation of bone marrow macrophages with amastigotes of *L. donovani* primed for an enhanced TNF-γ response to IFN-γ/LPS. Leishmanial parasites have been shown to enter human monocytes via fibronectin receptors,[54] a process which we found is inhibitable with the fibronectin analogue GRGDSPL for amastigotes of *L. donovani* (A. M. Cooper and J. M. Blackwell, unpublished observation).

Clearly, it will be important in future studies to determine whether *Nramp*-related differential amplification of priming/activation signals is related to specific interaction with β_1 integrins, or is more generally associated with multiple members of the β_2 integrin receptor family and/or with IFN-γ receptor activity. It will be important too to determine the role of specific tyrosine kinases, and whether interaction between the integrins and members of the family of actin-binding proteins, α-actinin, talin and vinculin, specifically known to interact with the cytoplasmic tail of the integrin receptors following ligation of fibronectin,[55] are involved in the *Nramp* response. Related proteins containing SH3-binding domains have been shown to associate with microtubules,[56] providing a critical link between signal transduction and endocytic pathways.[57] SH3 domains are responsible for cytoskeletal attachment and the targeting of signalling molecules to

specific subcellular locations.[14] In this way, the membrane-associated *Nramp* protein might act as a major focus for protein–protein interactions involved in initiation and amplification of signal transduction during macrophage priming/activation.

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